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(54) **CHEMICALLY MODIFIED POLYPEPTIDES**

(57) The present invention relates to a chemically modified polypeptide in which at least one of hydroxyl groups in the polypeptide molecule is modified with a polyalkylene glycol derivative; a method for producing the modified polypeptide; a method of treatment using the modified polypeptide; use of the modified polypeptide; a pharmaceutical preparation comprising the modified polypeptide; and a composition for treatment comprising the modified polypeptide.

EP 0 921 131 A1

Description

TECHNICAL FIELD

[0001] The present invention relates to a chemically modified polypeptide in which at least one of the hydroxyl groups in the polypeptide molecule is modified with a polyalkylene glycol derivative; a method for producing the modified polypeptide; a method of treating a patient having reduced granulocytes or thrombocytes using the modified polypeptide; a composition for the treatment comprising the modified polypeptide; and use of the modified polypeptide.

BACKGROUND ART

[0002] A chemically modified polypeptide in which at least one of amino groups, carboxyl groups, mercapto groups or guanidino groups in the polypeptide molecule is modified with a polyalkylene glycol derivative (WO 95/023165) and modification of free thiol groups of cysteine residues in the polypeptide molecule are known (EP 0668353). However, when at least one of such amino groups, carboxyl groups, mercapto groups, guanidino groups or free thiol groups of cysteine residues in the polypeptide molecule is modified with a polyalkylene glycol derivative, the activity of the polypeptide may be markedly or completely lost.

[0003] For example, the activity of interleukin-15 completely disappears when its amino group(s) are modified with polyethylene glycol [*J. Biol. Chem.*, 272:2312 (1997)].

[0004] Nothing is known about a chemically modified polypeptide in which at least one of the hydroxyl groups in the polypeptide molecule is modified with a polyalkylene glycol derivative.

[0005] Great attention has been directed toward the development of

(1) a method for the analysis of influences of hydroxyl groups upon the activity of a polypeptide, in a case where the hydroxyl group concerned is in the active site of the polypeptide,

(2) a novel chemical modification method which can avoid a probable case where the biological activity of a polypeptide is considerably spoiled when the polypeptide is treated by a conventional chemical modification method, and a chemically modified polypeptide obtained by the method, and

(3) a novel method which can improve resistance of polypeptide against protease, freezing-thawing and denaturing agents.

DISCLOSURE OF THE INVENTION

[0006] The present invention relates to a chemically modified polypeptide in which at least one of the hydroxyl groups in the polypeptide molecule is modified with a polyalkylene glycol derivative; a method for producing the modifying polypeptide; a method of treating a patient having reduced granulocytes or thrombocytes using the modifying polypeptide; use of the modifying polypeptide; a pharmaceutical preparation comprising the modifying polypeptide; and a composition for the treatment comprising the modifying polypeptide.

[0007] With regard to the polypeptide which can be used in the present invention, any polypeptide can be used so long as it contains a hydroxyl group and has a physiological activity or a pharmacological activity. Examples include those having an activity, such as asparaginase, glutaminase, uricase, superoxide dismutase, lactoferrin, streptokinase, plasmin, adenosine deaminase, interleukin-1 to 13, interleukin-15, interferon- α , interferon- β , interferon- γ , human granulocyte colony-stimulating factor (hereinafter referred to as "hG-CSF"), and the like.

[0008] Examples of a polypeptide having an hG-CSF activity include a polypeptide comprising the amino acid sequence represented by SEQ ID NO:1, a polypeptide comprising a partial amino acid sequence of the sequence, a polypeptide comprising an amino acid sequence in which some parts of amino acids of the sequence are substituted by different amino acids [*Nature*, 319:415 (1986), Japanese Published Unexamined Patent Application No. 267292/88, Japanese Published Unexamined Patent Application No. 299/88, WO 87/01132] and the like. Specific examples of the polypeptide comprising an amino acid sequence in which some parts of amino acids of the amino acid sequence represented by SEQ ID NO:1 are substituted by different amino acids (hG-CSF derivatives) are shown in Table 1.

Table 1

Position from the N-terminal amino acid (hG-CSF of SEQ ID NO:1)	Substituted amino acid in various hG-CSF derivatives												
	a)	b)	c)	d)	e)	f)	g)	h)	i)	j)	k)	l)	
1st (Thr)	*	Val	Cys	Tyr	Arg	*	Asn	Ile	Ser	*	Ala	*	
3rd (Leu)	Glu	Ile	Ile	Ile	Thr	Thr	Glu	Thr	Thr	*	Thr	*	
4th (Gly)	Lys	Arg	Arg	Arg	Arg	Arg	Arg	Arg	Arg	Arg	Tyr	*	
5th (Pro)	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	*	Arg	*
17th (Cys)	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser

*: Unsubstituted amino acid

[0009] Examples of the polyalkylene glycol derivative include polyethylene glycol derivatives, polypropylene glycol derivatives, polyethylene glycol-polypropylene glycol copolymer derivatives, and the like.

[0010] The chemically modified polypeptide of the present invention can be produced using a chemical modifying agent comprising the above polyalkylene glycol derivative, and compounds represented by the following formula (I) can be exemplified as preferred chemical modifying agents.

[0011] The compounds include polyalkylene glycol derivatives represented by

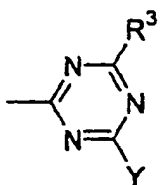


{wherein R^1 represents an alkyl group or an alkanoyl group; M represents

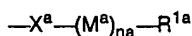
$-OCH_2CH_2-$, $-OCH_2CH_2CH_2-$ or

$-(OCH_2CH_2)_r-(OCH_2CH_2CH_2)_s-$

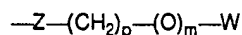
(wherein r and s are the same or different, and each represents an optionally changeable positive integer); n is an optionally changeable positive integer; X represents a bond, O, NH or S; and R^2 represents



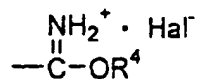
(wherein R^3 represents OH, halogen or



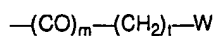
(wherein X^a , M^a , R^{1a} and na each has the same meanings as the above X, M, R^1 and n, respectively); and Y represents halogen or



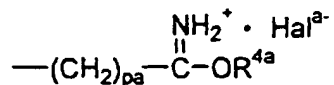
(wherein Z represents O, S or NH; and W represents a carboxyl group or a reactive derivative thereof, or



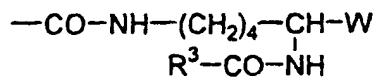
(wherein R^4 represents an alkyl group, and Hal represents halogen); p is an integer of 0 to 6; and m is 0 or 1],



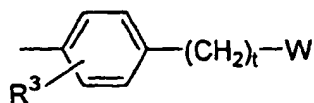
(wherein t is an integer of 0 to 6; and m and W have the same meanings as defined above)



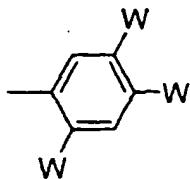
(wherein Hal^a , pa and R^{4a} each has the same meanings as the above Hal, p and R^4 , respectively),



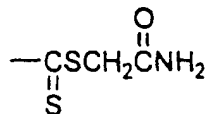
(wherein R^3 and W have the same meanings as defined above),



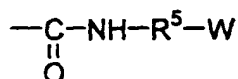
(wherein R^3 , t and W have the same meanings as defined above),



(wherein W has the same meaning as defined above),



or



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10 (wherein R⁵ represents a residue in which an amino group and a carboxyl group are removed from an amino acid; and W has the same meaning as defined above)).

[0012] With regard to the above compound represented by formula (I), examples of the alkyl group represented by R¹, R⁴ or the like include straight or branched alkyl groups having 1 to 18 carbon atoms, such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, pentyl, isopentyl, hexyl, isohexyl, heptyl, octyl, isooctyl, decyl, dodecyl, tetradecyl, hexadecyl, octadecyl, and the like; examples of the alkanoyl group represented by R¹ include straight or branched alkanoyl groups having 1 to 18 carbon atoms, such as formyl, acetyl, propionyl, butyryl, valeryl, pivaloyl, pentanoyl, lauroyl, myristoyl, palmitoyl, stearoyl, and the like; examples of the halogen represented by R³, Y, Hal or the like include chlorine, bromine and iodine atoms; examples of the reactive derivative of a carboxyl group represented by W or the like include acid halides, such as an acid chloride, an acid bromide, and the like, active esters, such as a p-nitrophenyl ester, an N-oxy succinimide ester, and the like, and mixed acid anhydrides with monoethyl carbonate, monoisobutyl carbonate and the like; and examples of the amino acid represented by R⁵ include glycine, L-alanine, L-valine, L-leucine, L-serine, D-alanine, D-valine, D-leucine, D-serine, β-alanine, and the like. The positive integer represented by n, r or s is 1 to 20,000, preferably 50 to 5,000 for n, and 1 to 5,000 for r and s.

[0013] The polyalkylene glycol derivatives have a molecular weight of 500 to 1,000,000, preferably 3,000 to 1,000,000.

[0014] A plurality of hydroxyl groups may be present in the polypeptide molecule, and chemical modification of at least one of these groups may be sufficient when the polypeptide is chemically modified.

[0015] Examples of the hydroxyl group in the polypeptide molecule include a hydroxyl group of a serine or threonine residue, preferably a hydroxyl group of a serine residue.

[0016] The polypeptide can be chemically modified by reacting a chemical modifying agent, such as a polyalkylene glycol derivative selected from a group consisting of polyethylene glycol derivatives, polypropylene glycol derivatives, polyethylene glycol-polypropylene glycol copolymer derivatives and the like, with a polypeptide having a hydroxyl group.

[0017] Examples of the method for reacting the hydroxyl group in a polypeptide molecule with a chemical modifying agent such as polyethylene glycol derivatives or polypropylene glycol derivatives include the methods described in Japanese Published Unexamined Patent Application No. 316400/89, *Biotech. Lett.*, **14**:559-564 (1992), *BIO/TECHNOLOGY*, **8**:343-346 (1990), and the like, and modified methods thereof. That is, specific examples of the methods which can be used include methods wherein a polyethylene glycol derivative or a polypropylene glycol derivative is added to an aqueous solution of a protein which has been adjusted to a pH of 6 to 10 in an amount of 1 to 200 moles per protein, and is allowed to react at a temperature of 0 to 37°C for 1 hour to 3 days.

[0018] Examples of the method for reacting the hydroxyl group in a polypeptide molecule with a polyethylene glycol-polypropylene glycol copolymer derivative include the methods described in Japanese Published Unexamined Patent Application No. 59629/84, Japanese Published Unexamined Patent Application No. 176586/85, WO 89/06546, EP 0539167A2, and the like, and modified methods thereof. That is, specific examples of the methods which can be used include methods wherein a chemical modifying agent selected from a group consisting of polyethylene glycol-polypropylene glycol copolymer derivatives is added to an aqueous solution of a protein which has been adjusted to a pH of 6 to 10 in an amount of 1 to 200 moles per protein, and is allowed to react at a temperature of 0 to 37°C for 1 hour to 3 days.

[0019] Modification of at least one hydroxyl group in the polypeptide molecule with a polyalkylene glycol derivative by the above method can provide

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(1) a method for the analysis of influences of the hydroxyl group upon the activity of a polypeptide, in a case where the hydroxyl group concerned is in the active site of the polypeptide,

(2) a novel chemical modification method which can avoid diminishing the biological activity of a polypeptide when the polypeptide is treated by a conventional chemical modification method, and a chemically modified polypeptide obtained by the method, and

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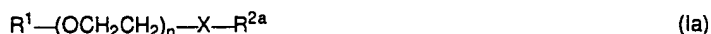
(3) a novel method which can improve a polypeptide's resistance to protease, freezing-thawing or denaturing agents, and a chemically modified polypeptide having improved resistance against protease, freezing-thawing or denaturing agents.

[0020] The chemically modified polypeptide of the present invention specifically described is an example in which a polypeptide having an hG-CSF activity is used as a polypeptide.

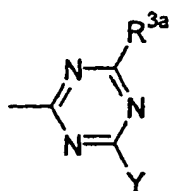
[0021] A chemically modified polypeptide in which at least one hydroxyl group in hG-CSF or a hG-CSF derivative is chemically modified with a chemical modifying agent represented by the following formula (la) or (lb) can be exemplified as the chemically modified polypeptide of the present invention.

Chemical modifying agent (la):

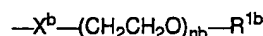
[0022]



{wherein R^1 , n and X have the same meanings as defined above; and R^{2a} represents



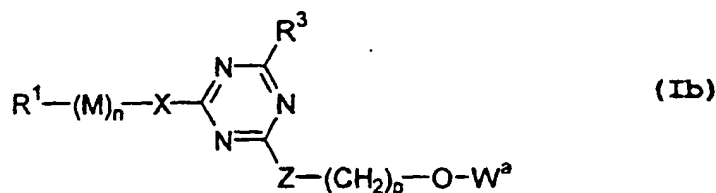
[wherein R^{3a} represents OH, halogen or



(wherein X^b , R^{1b} and nb each has the same meanings as the above X , R^1 and n , respectively))].

Chemical modifying agent (lb):

[0023]



(wherein R^1 , M , R^3 , Z , n and p have the same meanings as defined above; and W^a represents a carboxyl group or a reactive derivative thereof).

[0024] At least one molecule of polyethylene glycol derivatives, polypropylene glycol derivatives or polyethylene glycol-polypropylene glycol copolymer derivatives is bonded to a chemically modified hG-CSF or a chemically modified hG-CSF derivative. Thus, the chemically modified hG-CSF or the chemically modified hG-CSF derivative can be used as a mixture or by separating a compound to which one or more molecules are attached.

[0025] Separation of the chemically modified hG-CSF or the chemically modified hG-CSF derivative can be carried out using various types of chromatography, such as ion exchange chromatography, gel filtration chromatography, reverse phase chromatography, hydrophobic chromatography, and the like, and methods, such as ammonium sulfate fractionation and the like, which are generally used for the separation of long chain polypeptides and the like.

[0026] The degree of chemical modification can be confirmed by monitoring changes in the mobility of the chemically modified hG-CSF using a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method.

[0027] The polypeptide content according to the present invention can be measured by the following assay methods.

Assay method 1:

[0028] The polypeptide content is measured by the Lowry method [Lowry O.H. *et al.*, *J. Biol. Chem.*, 193:265 (1951)].

Assay method 2:

[0029] The polypeptide content is calculated by carrying out SDS-PAGE in accordance with the method of Laemmli [U.K. Laemmli, *Nature*, 227:680 (1970)], staining the polypeptide separated on the gel with Coomassie Brilliant Blue and then measuring it by a chromatoscanner (CS-930, Shimadzu Corp.).

[0030] The chemically modified polypeptide of the present invention can be used as such or in various dosage formulations.

[0031] The pharmaceutical preparations of the present invention can be produced by mixing an effective amount of a chemically modified polypeptide as the active ingredient uniformly with a pharmaceutically acceptable carrier. It is preferred that these pharmaceutical preparations are in a unit dosage form suitable for injection administration.

[0032] Injections can be prepared as solutions using a chemically modified polypeptide and a carrier comprising distilled water, a salt solution, a glucose solution or a mixture of a salt solution and a glucose solution. They are also prepared as solutions, suspensions or dispersions in the conventional way using appropriate auxiliaries. They can be also prepared as freeze-dried preparations by freeze-drying the solutions. Although freeze-drying conditions are not particularly limited, a freeze-dried product is generally obtained by freezing at -50°C or less for 1 to 5 hours, drying at a shelf temperature of -20°C to 0°C for 24 to 48 hours under a vacuum degree of 50 to 150 mTorr and then drying at a shelf temperature of 10°C to 30°C for 16 to 24 hours under a vacuum degree of 50 to 100 mTorr.

[0033] Also, the chemically modified polypeptide preparations can contain various generally used pharmaceutical carriers, fillers, diluents, stabilizers, adsorption preventing agents and the like.

[0034] In the case of a chemically modified polypeptide of the present invention, for example a neutrophil and thrombocyte growth enhancing preparation containing a chemically modified hG-CSF or a chemically modified hG-CSF derivative, its dose and its administration schedule are decided depending on its mode of administration, age and body weight of each patient, the disease to be treated and morbid state of each patient; however, a pharmaceutical preparation containing a chemically modified hG-CSF or a chemically modified hG-CSF derivative in an amount of 15 µg to 1.5 mg, preferably 25 to 500 µg, per adult is usually administered 1 to 7 times per week.

[0035] Examples of the administration method of the chemically modified polypeptide preparation of the present invention include intravenous injection, subcutaneous injection, and the like, as well as administration as suppositories or nasal drops.

[0036] Next, pharmacological activities of the chemically modified polypeptide of the present invention are described by Test Examples.

Test Example 1

Growth promoting activity of chemically modified hG-CSF and chemically modified hG-CSF derivatives upon mouse leukemia cell NFS60:

[0037] The activity of the G-CSF derivative, chemically modified hG-CSF derivative and chemically modified G-CSF obtained in Reference Example 1 and Examples 5, 14, 17 and 20, which will be described later, to enhance growth of mouse leukemia cell NFS60 [K. Holmes *et al.*, *Proc. Natl. Acad. Sci. USA*, 82:6687 (1985)] was measured in accordance with the method of Asano *et al.* [*Yakuri to Chiryō*, 19:2767 (1991)]. Each compound in respective concentrations shown in Tables 2-1 and 2-2 was allowed to act upon the cells, with the results also shown in Tables 2-1 and 2-2.

Table 2-1

Compound	Conc. (ng/ml)	NFS60 cell growth promoting activity (%) ^{*1}
hG-CSF derivative of Ref. Ex. 1	12.5	100
Mono-substituted 1 of Ex. 5	12.5	100
Mono-substituted 2 of Ex. 5	12.5	100
Di-substituted of Ex. 5	12.5	100
Tri-substituted 1 of Ex. 5	12.5	72
Tri-substituted 2 of Ex. 5	12.5	93
hG-CSF	5	100
Mono-substituted 1 of Ex. 14	5	86
Mono-substituted 2 of Ex. 14	5	81
Di-substituted of Ex. 14	5	81

^{*1}: Shown by a relative value (%) based on the activity (= 100) of the hG-CSF derivative (12.5 ng/ml) obtained in Reference Example 1 or of hG-CSF (5 ng/ml).

Table 2-2

Compound	Conc. (ng/ml)	NFS60 cell growth promoting activity (%) ^{*1}
hG-CSF derivative of Ref. Ex. 1	25	100
Tri-substituted 1 of Ex. 17	25	92
Tri-substituted 2 of Ex. 17	25	80
Di-substituted of Ex. 17	25	99
Tri-substituted 1 of Ex. 20	25	65
Tri-substituted 2 of Ex. 20	25	73
Di-substituted of Ex. 20	25	98

^{*1}: Shown by a relative value (%) based on the activity (= 100) of the hG-CSF derivative (25 ng/ml) obtained in Reference Example 1.

45 Test Example 2

Effect to promote recovery of thrombopenia in total body radiation mice:

[0038] Four animals per group of male BALB/c mice (6 weeks of age) were radiated to the total bodies (hereinafter referred to as "Rx") with 3 Gy per mouse by a ¹³⁷Cs radiation source (RI-433, manufactured by Toshiba) and then reared in a clean rack of a specified pathogen-free (SPF) rearing environment facility. They were freely provided with drinking water and feed. As a non-treated control group, mice with no radiation were reared in a manner similar thereto.

[0039] Each of chemically modified hG-CSF derivatives obtained in Example 4 which will be described later was dissolved in physiological saline, and the chemically modified hG-CSF derivative solution was subcutaneously administered to mice once on the next day of Rx in a dose of 5 µg/0.2 ml per animal.

[0040] Blood samples were periodically collected from the murine vein of eyeground to measure the number of platelets by an automatic blood cell counter (CC-180A, manufactured by Toa Iyo Denshi). The results are shown in Table 3.

[0041] In the mice treated with total body radiation of 3 Gy, a considerable decrease in the number of platelets was

observed, and the number of platelets became the lowest on 8 to 9 days after Rx and then gradually increased but did not recover to the level before the radiation treatment. On the other hand, decrease in the number of platelets was inhibited in the mice to which the chemically modified hG-CSF derivative was administered, and the number of platelets increased markedly on and after the 8th or 9th day and completely recovered on the 11th or 12th day to the same level before the radiation treatment. The similar effect was observed in a group in which the administration was carried out on the next day and 5th day after Rx.

Table 3

Chemically modified hG-CSF derivative	Average number of platelets (%) ^{*1}						
	Days after radiation	0	6	8	10	11	12
Not administered		100	58.9	26.6	35.1	38.3	45.1
Di-substituted of Ex. 4		100	55.1	30.5	62.7	98.5	103.6

^{*1}: Shown by a relative value (%) based on the average number of platelets (= 100) in the total body radiation-untreated control group.

Test Example 3

Leukocyte increasing action in mice:

[0042] Using SPF/VAF mice (BALB/cAnNCrj line, males, 8 weeks of age, 4 animals per group) which were preliminarily reared after purchase from Charles River Japan, leukocyte increasing activity in normal mice was confirmed.

[0043] Di-substituted obtained in Example 5 (0.34 mg/ml) was diluted to 100 or 10 µg/ml using physiological saline, and subcutaneously administered once at a rate of 10 µl/g (mouse weight). Thus, the dose was 1 or 0.1 mg/kg. As a control group, physiological saline was subcutaneously administered once. Blood samples were collected before the administration and periodically from the next day after the administration, and the number of peripheral blood cells was measured using an automatic blood cell counter (Sysmex F800).

[0044] As the results, the number of leukocytes in both administered groups increased to 2.4 to 2.6 times higher level than that of the control group 2 days after the administration. Thereafter, this drug effect attenuated in the 0.1 mg/kg administration group and returned to a similar level of the control group 4 days after the administration, but the number of leukocytes in the 1 mg/kg administration group continued to increase even after 2 days after administration and reached a level 3.5 times higher than that in the control group 4 days after administration. Thereafter, the number returned to a level similar to the control group on the 7th day after the administration.

[0045] Examples and Reference Examples are shown below.

BEST MODE FOR CARRYING OUT THE INVENTION

Example 1

Preparation of polyethylene glycol-modified hG-CSF derivative reaction solution:

[0046] The hG-CSF derivative prepared in Reference Example 1 was adjusted to 4.6 mg/ml using a phosphate buffer (pH 7.5), and 12 ml of the thus adjusted solution was mixed with 205.5 mg of N-hydroxysuccinimide ester of monomethoxypolyethylene glycol propionate (M-SPA-20,000, manufactured by Shearwater Polymer) and the mixture was stirred at 4°C for a whole day and night to prepare a polyethylene glycol-modified hG-CSF derivative reaction solution (1).

Example 2

Preparation of polyethylene glycol-modified hG-CSF derivative reaction solution:

[0047] The hG-CSF derivative prepared in Reference Example 1 was adjusted to 4.6 mg/ml using a phosphate buffer (pH 7.5), and 12 ml of the thus adjusted solution was mixed with 469.8 mg of N-hydroxysuccinimide ester of carboxymethyl-monomethoxypolyethylene glycol (M-SCM-20,000, manufactured by Shearwater Polymer) and the mixture was stirred at 4°C for a whole day and night to prepare a polyethylene glycol-modified hG-CSF derivative reaction solution

(2).

Example 3

5 Preparation of polyethylene glycol-modified hG-CSF derivative reaction solution:

[0048] The hG-CSF derivative prepared in Reference Example 1 was adjusted to 4.6 mg/ml using a phosphate buffer (pH 7.5), and 6.6 ml of the thus adjusted solution was mixed with 96.9 mg of N-hydroxysuccinimide ester of monomethoxypolyethylene glycol propionate (M-SSPA-20,000, manufactured by Shearwater Polymer) and the mixture was stirred
10 at 4°C for a whole day and night to prepare a polyethylene glycol-modified hG-CSF derivative reaction solution (3).

Example 4

Isolation of polyethylene glycol-modified component:

15 [0049] The polyethylene glycol-modified hG-CSF derivative reaction solution (2) prepared in Example 2 (protein content, 55 mg) was diluted 5 times with a 20 mM acetate buffer (pH 4.5) containing 150 mM of sodium chloride and passed through a column packed with Sephacryl S-400 (manufactured by Pharmacia) which had been equilibrated in advance with the same buffer, and the eluates were fractionated. By this operation, a fraction mainly containing a component
20 modified with two molecules of polyethylene glycol (Di-substituted) and a crude fraction mainly containing a component modified with one molecule of polyethylene glycol (Mono-substituted) were obtained.

[0050] Each of these fractions was passed through a column packed with TSK gel G4000SW_{XL} (7.8 mm I.D. × 300 mm, manufactured by Tosoh Corp.) to obtain a fraction containing Di-substituted or Mono-substituted. Thereafter, each of these fractions was purified using SP-5PW (21.5 mm I.D. × 150 mm, manufactured by Tosoh Corp.) under the follow-
25 ing conditions to obtain one species of the main component of Di-substituted (0.39 mg/ml × 3.0 ml) and one species of the main component of Mono-substituted (0.55 mg/ml × 3.0 ml).

SP-5PW purification conditions:

30 [0051]

Column: SP-5PW (21.5 mm I.D. × 150 mm) (manufactured by Tosoh Corp.)

Detection: 280 nm

Solution A: 20 mM acetate buffer (pH 4.5)

35 Solution B: 20 mM acetate buffer (pH 4.5) containing 0.5 M sodium chloride

Elution: linear density gradient elution from solution A to solution B

Example 5

40 Isolation of polyethylene glycol-modified component:

[0052] The polyethylene glycol-modified hG-CSF derivative reaction solution (1) prepared in Example 1 (protein content, 55 mg) was diluted 5 times with a 20 mM acetate buffer (pH 4.5) containing 150 mM of sodium chloride and passed through a column packed with Sephacryl S-400 (manufactured by Pharmacia) which had been equilibrated in advance
45 with the same buffer, and the eluates were fractionated. By this operation, a crude fraction containing a component modified with three molecules of polyethylene glycol (Tri-substituted), a crude fraction containing a component modified with two molecules of polyethylene glycol (Di-substituted) and a crude fraction containing a component modified with one molecules of polyethylene glycol (Mono-substituted) were obtained.

[0053] Each of these fractions was passed through a column packed with TSK gel G4000SW_{XL} (7.8 mm I.D. × 300 mm, manufactured by Tosoh Corp.) to obtain a fraction containing Tri-substituted, Di-substituted or Mono-substituted. Thereafter, each of these fractions was purified using a cation exchange column, SP-5PW (manufactured by Tosoh Corp.), under the same conditions described in Example 4 to obtain two species of the main component of Tri-substituted (Tri-substituted 1: 1.4 mg/ml × 0.5 ml, Tri-substituted 2: 1.0 mg/ml × 0.8 ml), one species of the main component of Di-substituted (0.34 mg/ml × 3.0 ml) and two species of the main components of Mono-substituted (Mono-substituted
50 1: 0.48 mg/ml × 0.4 ml, Mono-substituted 2: 1.58 mg/ml × 0.5 ml).

[0054] As will be described in later examples, the hydroxyl group of the 66 position Ser counting from the N-terminus of Mono-substituted 1 and the N-terminal Met of Mono-substituted 2 were modified with one molecule of polyethylene glycol.

Example 6

Isolation of polyethylene glycol-modified component:

[0055] The polyethylene glycol-modified hG-CSF derivative reaction solution (3) prepared in Example 3 (protein content, 30 mg) was diluted 5 times with a 20 mM acetate buffer (pH 4.5) containing 150 mM of sodium chloride and passed through a column packed with Sephacryl S-400 (manufactured by Pharmacia) which had been equilibrated in advance with the same buffer, and the eluates were fractionated. By this operation, a crude fraction containing a component modified with two molecules of polyethylene glycol (Di-substituted) was obtained.

[0056] This fraction was passed through a column packed with TSK gel G4000SW_{XL} (7.8 mm I.D. × 300 mm, manufactured by Tosoh Corp.) to obtain a fraction containing Di-substituted.

[0057] The thus obtained fraction was purified using a cation exchange column, SP-5PW (manufactured by Tosoh Corp.) under the same conditions described in Example 4 to obtain one species of the main component of Di-substituted (0.43 mg/ml × 3.0 ml).

Example 7

Peptide mapping of hG-CSF derivative:

[0058] The hG-CSF derivative prepared in Reference Example 1 was adjusted to 2 mg/ml using a phosphate buffer (pH 7.5), a 1.0 ml portion of the thus adjusted solution was treated with V8 protease (manufactured by Seikagaku Kogyo) under the conditions described in "High Performance Liquid Chromatography of Protein and Peptide (II), Kagaku Zokan 117 (153-160 (1990); published by Kagaku Dojin)" and then a 50 µl portion of the thus treated solution was injected into an HPLC to carry out HPLC analysis under the following conditions.

Conditions for HPLC analysis:

[0059]

Column: PROTEIN & PEPTIDE C18 (4.6 mm I.D. × 250 mm, VYDAC)

Solution A: 0.1% trifluoroacetic acid

Solution B: 90% acetonitrile containing 0.1% trifluoroacetic acid

Elution: linear density gradient elution from solution A to solution B

Detection: 215 nm

Flow rate: 0.5 ml/min

[0060] Nine peaks were found on the analytical pattern of the hG-CSF derivative digested with V8 protease. When each of the peaks was separated and its molecular weight analysis was carried out, these peaks were assigned as shown in Table 4.

Table 4

Peptide mapping of hG-CSF derivative by V8 protease		
Peak No.	Peptide fragment	Molecular weight analyzed (calculated) *(Na addition)
1	94 - 98	524.5* (502)
2	20 - 33	1512.8 (1514)
3	34 - 46	1648.8 (1649)
4	163 - 174	1438.9 (1440)
5	-1 - 19	2241.7 (2241)
6	124 - 162	4028.0 (4029)
7	105 - 123	2263.8* (2240)

Table 4 (continued)

Peptide mapping of hG-CSF derivative by V8 protease		
Peak No.	Peptide fragment	Molecular weight analyzed (calculated) *(Na addition)
8	47 - 93	5060.5 (5060)
9	99 - 123	2836.9 (2836)

Example 8

Estimation of polyethylene glycol binding position by peptide mapping:

[0061] Each of Mono-substituted 1, Mono-substituted 2, Di-substituted, Tri-substituted 1 and Tri-substituted 2 obtained in Example 5 was digested with a protease by a procedure similar to the hG-CSF derivative peptide mapping described in Example 7.

[0062] As the results, disappearance or considerable reduction of specified peaks was found in Mono-substituted 1, Di-substituted, Tri-substituted 1 and Tri-substituted 2 obtained in Example 5 (Table 5).

Table 5

Peptide mapping of purified polyethylene glycol-modified hG-CSF derivative component (comparison with unmodified hG-CSF derivative)	
Component	Disappeared or markedly reduced peak No.
Mono-substituted 1 of Ex. 5	8
Mono-substituted 2 of Ex. 5	5
Di-substituted of Ex. 5	5, 8
Tri-substituted 1 of Ex. 5	3, 5, 8
Tri-substituted 2 of Ex. 5	3, 5, 8

[0063] It is believed that the HPLC peaks of the polyethylene glycol-linked peptide fragments will change when compared with the analytical pattern of the hG-CSF derivative of Example 7. Based on the results of Table 4, it was estimated that the binding position of polyethylene glycol was at least a peptide residue (including an N-terminal amino group) corresponding to a fragment of -1 to 19 amino acid residues (peak 5) of the hG-CSF derivative in the case of Mono-substituted 2, Di-substituted, Tri-substituted 1 and Tri-substituted 2 shown in Example 5.

[0064] With regard to Mono-substituted 2, it was found that the N-terminal Met residue of Mono-substituted 2 was modified with one molecule of polyethylene glycol, because its N-terminal sequence was not observed when the sequence was measured using a protein sequencer PPSQ-10 manufactured by Shimadzu Corp.

[0065] With regard to Tri-substituted 1 and Tri-substituted 2, it was estimated that polyethylene glycol was also bound to at least a peptide residue (including an amino group of Lys) corresponding to a fragment of 34 to 46 amino acid residues (peak 3) of the hG-CSF derivative.

[0066] In addition, since the peak 8 disappeared or markedly reduced in each of Mono-substituted 1, Di-substituted, Tri-substituted 1 and Tri-substituted 2, it was estimated that polyethylene glycol was also bound to a peptide residue (excluding free amino groups of lysine and the like) corresponding to a fragment of 47 to 93 amino acid residues of each of these components.

Example 9

Estimation of polyethylene glycol binding position by peptide mapping:

[0067] The fraction of Di-substituted obtained in Example 6 was digested with a protease by a procedure similar to

the hG-CSF derivative peptide mapping described in Example 7.

[0068] When compared with the analytical pattern of the hG-CSF derivative of Example 7, disappearance or considerable reduction of the specified peaks shown in Table 6 was found in Di-substituted obtained in Example 6.

Table 6

Peptide mapping of purified polyethylene glycol-modified hG-CSF derivative component (comparison with unmodified hG-CSF derivative)	
Component	Disappeared or markedly reduced peak No.
Di-substituted of Ex. 5	5, 8

[0069] It is predicted that the HPLC peaks of the polyethylene glycol-linked peptide fragments will change when compared with the analytical pattern of the hG-CSF derivative of Example 7. Based on the results of Table 5, similar to the case of Example 8, it was estimated that binding positions of polyethylene glycol were a peptide residue (including an N-terminal amino group) corresponding to a fragment of -1 to 19 amino acid residues and a peptide residue (excluding free amino groups of lysine and the like) corresponding to a fragment of 47 to 93 amino acid residues of the hG-CSF derivative in the case of Di-substituted shown in Example 6.

Example 10

Estimation of polyethylene glycol binding position by peptide mapping:

[0070] The binding position of polyethylene glycol on Di-substituted obtained by a procedure similar to that of Example 4 was examined by peptide mapping in a manner similar to those in Examples 7 and 8. It was estimated that polyethylene glycol was also bound to a peptide residue (including an N-terminal amino group) corresponding to a fragment of -1 to 19 amino acid residues and a peptide residue (excluding free amino groups of lysine and the like) corresponding to a fragment of 47 to 93 amino acid residues of the hG-CSF derivative in the case of Di-substituted shown in Example 4.

Example 11

Purification of polyethylene glycol-modified peptide:

[0071] A fragment of polyethylene glycol-bound peptide was isolated from Di-substituted component by the following procedure in order to examine polyethylene glycol binding amino acid residues in the peptide fragment corresponding to the 47 to 93 position amino acid residues containing no free amino groups, of the hG-CSF derivative or hG-CSF, which was the polyethylene glycol binding position confirmed in Di-substituted of Examples 4, 5 and 6, Tri-substituted 1 and Tri-substituted 2 of Example 5, Mono-substituted 1 of Example 5, Mono-substituted 1 and Di-substituted of Example 14, Di-substituted, Tri-substituted 1 and Tri-substituted 2 of Example 17 or Di-substituted, Tri-substituted 1 and Tri-substituted 2 of Example 20.

[0072] That is, 30 ml of a fraction containing Di-substituted as the main component (protein concentration, 2.2 mg/ml) was obtained by a cation exchange chromatography column, SP-5PW, in a manner similar to that in Example 5 from a polyethylene glycol-modified hG-CSF derivative reaction solution prepared in a manner similar to that in Example 1. Next, this was digested with thermolysin (manufactured by Sigma). Using a gel filtration column (Sephacryl S-300, manufactured by Pharmacia), the enzyme reaction solution was fractionated to obtain the desired polyethylene glycol-bound peptide fraction (about 30 mg).

[0073] The result of mass spectrometry (MALDI-TOF MS) of the peptide fraction was 22155.89 (M+H), and the result of amino acid analysis showed Ser 2.7 (3), Pro 1.0 (1), Leu 1.0 (1) and Cys 0.9 (1).

[0074] Based on the results of mass spectrometry and amino acid analysis, it was estimated that the sample isolated by the above procedure was a fragment in which polyethylene glycol was bound to a peptide of position 61 residue to the 66 position residue [LeuSerSerCysProSer (leucyl-seryl-seryl-S-amidomethylcysteinyl-prolyl-serine) residue] of the hG-CSF derivative.

Example 12

Determination of polyethylene glycol binding position by $^1\text{H-NMR}$:

- 5 [0075] A peptide, LeuSerSerCysProSer (leucyl-seryl-seryl-cysteinyl-prolyl-serine) corresponding to the 61 to 66 position amino acid residues of the hG-CSF derivative was synthesized by a peptide solid phase synthesis method (PSSM 8, Shimadzu Corp.) and amidomethylated under the conditions shown in "High Performance Liquid Chromatography of Protein and Peptide (II), Kagaku Zokan 117 (153-160 (1990); published by Kagaku Dojin)" and then the thus amidomethylated peptide (leucyl-seryl-seryl-S-amidomethylcysteinyl-prolyl-serine) was purified by reverse phase HPLC. A
- 10 0.6 mg portion of the amidomethylated peptide was dissolved in deuterium-substituted dimethyl sulfoxide (d_6 -DMSO) to carry out $^1\text{H-NMR}$ analysis (500 MHz). In a similar manner, $^1\text{H-NMR}$ analysis was carried out using 20 mg of the polyethylene glycol-bound peptide obtained in Example 11. Chemical shift of protons (other than those originating from polyethylene glycol) observed by each analysis is shown in Tables 7 and 8. From the results, all of the protons originating from the γOH groups of three Ser residues were confirmed in the amidomethylated peptide obtained by synthesis.
- 15 On the other hand, some phenomena were observed in the polyethylene glycol-bound peptide obtained in Example 11, in addition to the signal of protons originating from polyethylene glycol, that the proton originating from γOH group of the 66 position Ser residue of the hG-CSF derivative was not observed, that β proton of the same Ser residue was shifted to about 0.6 ppm-lower magnetic field and that amido proton of the same Ser residue gave a broad signal in comparison with other amido protons. On the basis of the above results, it was confirmed that the binding position of polyethylene
- 20 glycol was the 66 position Ser residue of the hG-CSF derivative.

Table 7

Chemical shift (ppm) of protons in NMR analysis of amidomethylated peptide synthesized				
Residue	NH	C α H	C β H	Others
Leu 61	8.06	3.86	1.52, 1.57	γH 1.67, δCH_3 0.92
Ser 62	8.66	4.47	3.57, 3.64	γOH 5.13
	<i>8.66</i>	<i>4.49</i>	<i>3.63</i>	<i>γOH5.04</i>
Ser 63	8.03	4.32	3.58	γOH 4.85
	<i>8.06</i>	<i>4.32</i>	<i>3.57</i>	<i>γOH4.80</i>
Cys 64	8.19	4.68	2.63, 2.96	CH_2 3.12, NH_2 7.05, 7.42
	<i>7.97</i>	<i>4.60</i>	<i>2.63, 2.90</i>	<i>$\text{CH}_2$3.12, $\text{NH}_2$7.10, 7.45</i>
Pro 65		4.42	1.91, 2.02	γH 1.86, δH 3.61
		<i>4.80</i>	<i>1.98, 2.20</i>	<i>γH1.83, δH3.45, 3.40</i>
Ser 66	7.98	4.23	3.60, 3.70	γOH 4.94
	<i>8.40</i>	<i>4.24</i>	<i>3.80, 3.72</i>	<i>γOH4.90</i>
The italic type represents Cis-Pro.				

Table 8

Chemical shift (ppm, excluding the polyethylene glycol moiety) of protons in NMR analysis of polyethylene glycol-modified peptide isolated from polyethylene glycol-modified hG-CSF derivative				
Residue	NH	C α H	C β H	Others
Leu 61		3.66	1.44, 1.56	γH 1.70, δCH_3 0.90
		3.73	1.47, 1.60	γH 1.67, δCH_3 0.90

Table 8 (continued)

Chemical shift (ppm, excluding the polyethylene glycol moiety) of protons in NMR analysis of polyethylene glycol-modified peptide isolated from polyethylene glycol-modified hG-CSF derivative				
Residue	NH	C α H	C β H	Others
Ser 62	8.51 <i>8.90</i>	4.43 <i>4.37</i>	3.57, 3.64 <i>3.64</i>	γ OH5.17
Ser 63	8.03 <i>8.06</i>	4.30 <i>4.21</i>	3.60 <i>3.58, 3.63</i>	γ OH4.85
Cys 64	8.18 <i>8.08</i>	4.68 <i>4.62</i>	2.68, 3.03 <i>2.67, 2.90</i>	CH ₂ 3.12, NH ₂ 7.02, 7.43 <i>CH₂3.12, NH₂7.02, 7.62</i>
Pro 65		4.33 <i>4.60</i>	1.87, 2.02 <i>2.12, 2.04</i>	γ H1.86, δ H3.67 <i>γH1.72, 1.83, δH3.40, 3.52</i>
Ser 66	7.88 <i>7.66</i>	4.28 <i>4.21</i>	4.17, 4.32 <i>4.16, 4.46</i>	
The italic type represents Cis-Pro.				

Example 13

Preparation of polyethylene glycol-modified hG-CSF reaction solution:

[0076] A 4.0 ml portion of a 4.0 mg/ml solution of hG-CSF prepared using a phosphate buffer (pH 7.5) was mixed with 83 mg of N-hydroxysuccinimide ester of monomethoxypolyethylene glycol propionate (M-SPA-20,000, manufactured by Shearwater Polymer) and the mixture was stirred at 4°C for a whole day and night.

Example 14

Isolation of polyethylene glycol-modified component:

[0077] The polyethylene glycol-modified reaction solution prepared in Example 13 (protein content, 16 mg) was diluted 5 times with a 20 mM acetate buffer (pH 4.5) containing 150 mM of sodium chloride and fractionated using a column packed with Sephacryl S-400 (manufactured by Pharmacia) which had been equilibrated in advance with the same buffer. By this operation, a fraction mainly containing a component modified with two molecules of polyethylene glycol (Di-substituted) and a crude fraction mainly containing a component modified with one molecule of polyethylene glycol (Mono-substituted) were obtained. Each of these fractions was applied to a column packed with TSK gel G4000SW_{XL} (7.8 mm I.D. × 300 mm, manufactured by Tosoh Corp.) to obtain a fraction containing Di-substituted or Mono-substituted. Thereafter, each of these fractions was purified using SP-5PW (21.5 mm I.D. × 150 mm, manufactured by Tosoh Corp.) to obtain one species of the main component of Di-substituted (0.61 mg/ml × 0.6 ml) and two species of the main component of Mono-substituted (Mono-substituted 1: 0.46 mg/ml × 1.0 ml, Mono-substituted 2: 0.61 mg/ml × 0.7 ml).

Example 15

Estimation of polyethylene glycol binding position by peptide mapping:

[0078] Each of Mono-substituted 1, Mono-substituted 2 and Di-substituted obtained in Example 14 was digested with a protease by a procedure similar to the hG-CSF derivative peptide mapping described in Example 7.

[0079] As the results, disappearance or considerable reduction of specified peaks was found when compared with the analytical pattern of hG-CSF as was found in Example 8.

[0080] According to a method similar to that in Example 8, the binding position of polyethylene glycol was estimated as follows.

Mono-substituted 1:

[0081] The position was within a peptide fragment (not including free amino groups of lysine and the like) corresponding to the 47 to 93 position residues of the amino acid sequence represented by SEQ ID NO:1, and it was confirmed according to the method described in Example 12 that the hydroxyl group of the 66 position Ser counting from the N-terminus was modified with one molecule of polyethylene glycol.

Mono-substituted 2:

[0082] The position was within a peptide fragment (including an N-terminal amino group) corresponding to the -1 to 19 position residues of the amino acid sequence represented by SEQ ID NO:1, and it was confirmed according to the method described in Example 7 that the N-terminal Met was modified with one molecule of polyethylene glycol.

Di-substituted:

[0083] A peptide fragment (including an N-terminal amino group) corresponding to the -1 to 19 position residues, and a peptide fragment (not including free amino groups of lysine and the like residues) corresponding to the 47 to 93 position residues, of the amino acid sequence represented by SEQ ID NO:1.

Example 16

Preparation of polyethylene glycol-modified hG-CSF derivative reaction solution:

[0084] The hG-CSF derivative prepared in Reference Example 1 was adjusted to 0.9 mg/ml using a phosphate buffer (pH 7.2) to obtain 600 ml of the adjusted solution. While cooling in an ice bath, the thus obtained solution was mixed with 8.7 g of N-hydroxysuccinimide ester of 2,4-bis(o-methoxypolyethylene glycol)-6-(3-carboxypropylamino)-s-triazine obtained by a method similar to that in Reference Example 2, and the mixture was stirred at 4°C for 48 hours to prepare a polyethylene glycol-modified hG-CSF derivative reaction solution (4).

Example 17

Isolation of polyethylene glycol-modified component:

[0085] The polyethylene glycol-modified hG-CSF derivative reaction solution (4) prepared by a method similar to that in Example 16 (protein content, 40 mg) was passed through a column packed with Sephacryl S-300 (manufactured by Pharmacia) which had been equilibrated in advance with a 20 mM acetate buffer (pH 4.5) containing 150 mM of sodium chloride, and the eluates were fractionated. By this operation, a crude fraction containing a component modified with three molecules of polyethylene glycol (Tri-substituted) and a crude fraction containing a component modified with two molecules of polyethylene glycol (Di-substituted) were obtained.

[0086] Each of these fractions was purified using a cation exchange column SP-5PW (manufactured by Tosoh Corp.) in a manner similar to that in Example 4 to obtain two species of the main components of Tri-substituted (Tri-substituted 1: 0.49 mg/ml × 1.3 ml, Tri-substituted 2: 0.57 mg/ml × 1.5 ml) and one species of the main component of Di-substituted (1.94 mg/ml × 1.2 ml).

Example 18

Estimation of polyethylene glycol binding position by peptide mapping:

[0087] Polyethylene glycol-binding positions of Di-substituted, Tri-substituted 1 and Tri-substituted 2 obtained in Example 17 were examined by a procedure similar to the peptide mapping described in Examples 7 and 8.

[0088] Also in Di-substituted, Tri-substituted 1 and Tri-substituted 2 obtained in Example 17, it was estimated that polyethylene glycol was bound to a peptide fragment (including an N-terminal amino group) corresponding to the -1 to 19 position residues of the hG-CSF derivative and to a peptide fragment (not including free amino groups of lysine and the like) corresponding to the 47 to 93 position residues of the hG-CSF derivative. In Tri-substituted 1 and Tri-substituted 2, it was estimated that polyethylene glycol was also bound to at least a peptide fragment (including an amino group of Lys) corresponding to the 34 to 46 position residues (peak 3) of the hG-CSF derivative.

Example 19

Preparation of polyethylene glycol-modified hG-CSF derivative reaction solution:

[0089] The hG-CSF derivative prepared in Reference Example 1 was adjusted to 0.9 mg/ml using a phosphate buffer (pH 7.3) to obtain 560 ml of the adjusted solution. While cooling in an ice bath, the thus obtained solution was mixed with 22.4 g of N-hydroxysuccinimide ester of 2,4-bis(o-methoxypolyethylene glycol)-6-(3-carboxypropylamino)-s-triazine obtained by a method described similar to that in Reference Example 4, and the mixture was stirred at 4°C for 48 hours to prepare a polyethylene glycol-modified hG-CSF derivative reaction solution (5).

Example 20

Isolation of polyethylene glycol-modified component:

[0090] The polyethylene glycol-modified hG-CSF derivative reaction solution (5) prepared by a method similar to that in Example 19 (protein content, 15 mg) was passed through a column packed with Sephacryl S-400 (manufactured by Pharmacia) which had been equilibrated in advance with a 20 mM acetate buffer (pH 4.5) containing 150 mM of sodium chloride, and the eluates were fractionated. By this operation, a crude fraction containing a component modified with three molecules of polyethylene glycol (Tri-substituted) and a crude fraction containing a component modified with two molecules of polyethylene glycol (Di-substituted) were obtained.

[0091] Each of these fractions was purified using a cation exchange column SP-5PW (manufactured by Tosoh Corp.) in a manner similar to that in Example 4 to obtain two species of the main components of Tri-substituted (Tri-substituted 1: 3.53 mg/ml \times 0.4 ml, Tri-substituted 2: 0.26 mg/ml \times 2.1 ml) and one species of the main component of Di-substituted (0.84 mg/ml \times 1.2 ml).

Example 21

Estimation of polyethylene glycol binding position by peptide mapping:

[0092] Polyethylene glycol-binding positions of Di-substituted, Tri-substituted 1 and Tri-substituted 2 obtained in Example 20 were examined by a procedure similar to the peptide mapping described in Examples 7 and 8. Also in Di-substituted, Tri-substituted 1 and Tri-substituted 2 obtained in Example 20, it was estimated that polyethylene glycol was bound to a peptide fragment (including an N-terminal amino group) corresponding to the -1 to 19 position residues of the hG-CSF derivative and to a peptide fragment (not including free amino groups of lysine and the like) corresponding to the 47 to 93 position residues of the hG-CSF derivative. In Tri-substituted 1 and Tri-substituted 2, it was estimated that polyethylene glycol was also bound to at least a peptide fragment (including an amino group of Lys) corresponding to the 34 to 46 position residues of the hG-CSF derivative.

Example 22

Protease resistance of chemically modified polypeptide:

[0093] A 0.5 ml portion of each of the two species of Mono-substituted components obtained in Example 14 was passed through a gel filtration column [Sephadex G-25 (NAP-5, manufactured by Amersham-Pharmacia)] which had been equilibrated with a 10 mM phosphate buffer (pH 5.0), and the eluates were recovered in 1.0 ml portions.

[0094] Each of the thus recovered fractions was diluted with a 10 mM phosphate buffer (pH 5.0) to adjust the protein concentration to 0.2 mg/ml.

[0095] A 2 ml portion of each of the thus diluted fractions was mixed with 0.2 mg/ml of thermolysin (enzyme/substrate ratio = 1/50) and the mixture was allowed to react at 30°C.

[0096] Samples were periodically collected from the reaction solutions in 100 μ l portions and mixed with 2 μ l of acetic acid to terminate the reaction.

[0097] Using 50 μ l of each of the reaction-terminated samples, HPLC analysis was carried out under the following conditions.

HPLC analysis conditions:

[0098]

5 Column: TSK gel G4000SW_{XL} (7.8 mm I.D. × 300 mm) (manufactured by Tosoh)
 Detection: 280 nm
 Mobile phase: 150 mM NaCl/20 mM sodium acetate buffer (pH 4.5)
 Flow rate: 0.8 ml/min

10 [0099] Mono-substituted's 1 and 2 were eluted at a retention time of 12 minutes. When these Mono-substituted's were hydrolyzed by thermolysin, the detection peak found at the position of 12 minutes decreases so that the degree of hydrolysis of these Mono substituted's by thermolysin could be confirmed.

[0100] Table 9 shows periodic changes in the residual ratio of Mono-substituted's calculated from the decreasing ratio of the detection peak found at the position of 12 minutes.

Table 9

Stability against thermolysin		
Reaction time (hr)	Mono-substituted 1 of Ex. 14	Mono-substituted 2 of Ex. 14
0	100	100
15	95	72
23	89	68

[0101] It was found from the results shown in Table. 9 that Mono-substituted 1 in which Ser was modified with polyethylene glycol was more resistant to thermolysin than Mono-substituted 2 in which the N-terminus was modified.

Example 23

Stability of chemically modified polypeptide against freezing-thawing:

35 [0102] A 1 ml portion of each of the two species of Mono-substituted components obtained in Example 5 was passed through two gel filtration columns [Sephadex G-25 (NAP-10, manufactured by Amersham-Pharmacia)] which had been equilibrated with a 5 mM sodium acetate buffer (pH 4.5) or a 5 mM sodium acetate buffer (pH 5.0), and the eluates from each column were recovered in 1.5 ml portions.

[0103] The protein concentration of each of the thus recovered fractions was adjusted to 300 µg/ml.

40 [0104] Each of the thus adjusted fractions was dispensed in 500 µl portions, frozen at -30°C and then melted in a water bath at room temperature.

[0105] This freezing-thawing treatment was repeated four times, and then the remaining amount (recovery yield) of each modified polypeptide was measured under the gel filtration HPLC conditions described in Example 22.

[0106] The results are shown in Table 10.

Table 10

Polyethylene glycol-modified product	Recovery yield (%) after four freezing-melting treatments	
	pH 4.5	pH 5.0
Mono-substituted 1 of Ex. 5	100	101
Mono-substituted 2 of Ex. 5	91.3	85.5

55 [0107] It was found from the results shown in Table 10 that Mono-substituted 1 in which Ser was modified with polyethylene glycol was more stable against freezing-thawing than Mono-substituted 2 in which the N-terminus was modified.

Example 24

In vitro activity of chemically modified polypeptide:

- 5 [0108] The activity of the two species of Mono-substituted components obtained in Example 14 to enhance growth of mouse leukemia cell NFS60 [K. Holmes *et al.*, *Proc. Natl. Acad. Sci. USA*, 82:6687 (1985)] was measured in accordance with the method of Asano *et al.* [Yakuri to Chiryo, 19:2767 (1991)] by the following serial dilution method.
- [0109] A suspension of the cells washed with G-CSF (-) medium was dispensed in 50 μ l portions into wells of a 96 well plate.
- 10 [0110] Mono-substituted 1 obtained in Example 14 was adjusted to 25 ng/ml, and a 50 μ l portion of the adjusted solution was added to the first well and thoroughly mixed to adjust the solution to 12.5 ng/ml.
- [0111] A 50 μ l portion of the solution was removed from the well and added to the second well and thoroughly mixed to adjust the solution to 6.25 ng/ml. By repeating this procedure, 11 steps of two-fold dilution series were prepared.
- [0112] In the same manner, two-fold dilution series were prepared using Mono-substituted 2 (25 ng/ml) obtained in
- 15 Example 14 and a standard solution (5 ng/ml) containing the hG-CSF derivative of Reference Example 1. By this method, dilution series of Mono-substituted 2 from 12.5 ng/ml and dilution series of the standard solution from 2.5 ng/ml were prepared in 50 μ l portions in respective wells.
- [0113] The growth activity of NFS60 cells was measured three times for each of the sample solutions and the standard solutions, and relative activity of each Mono-substituted was calculated based on the activity (= 100) of the standard
- 20 solution.
- [0114] Mono-substituted 1 showed 1.06 to 1.13 times higher activity than Mono-substituted 2.

Reference Example 1

- 25 [0115] In accordance with the method described in Reference Example 3 of Japanese Examined Patent Application No. 096558/95, a hG-CSF derivative (compound k in Table 1) was obtained from hG-CSF having the amino acid sequence shown in SEQ ID NO:1 by replacing the 1 position threonine with alanine, the 3 position leucine with threonine, the 4 position glycine with tyrosine, the 5 position proline with arginine and the 17 position cystine with serine.
- [0116] That is, a strain of *Escherichia coli*, W3110 strA (*Escherichia coli* ECfBD28, FERM BP-1479), which has a plasmid pCfBD28 containing a DNA fragment coding for the above-described hG-CSF derivative was cultured at 37°C
- 30 for 18 hours in LG medium [prepared by dissolving 10 g of Bacto Tryptone, 5 g of Yeast Extract, 5 g of sodium chloride and 1 g of glucose in 1 liter of water and adjusting its pH to 7.9 with NaOH], a 5 ml portion of the cultured broth was inoculated into 100 ml of MCG medium (0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.5% sodium chloride, 0.5% casamino acid, 1 mM MgSO₄ and 4 μ g/ml of vitamin B1, pH 7.2) containing 25 μ g/ml of tryptophan and 50 μ g/ml of ampicillin and the
- 35 mixture was cultured at 30°C for 4 to 8 hours. Then, 10 μ g/ml of a tryptophan inducer 3 β -indoleacrylic acid (hereinafter referred to as "IAA") was added to the medium and the culturing was continued for additional 2 to 12 hours. The resulting culture broth was centrifuged at 8,000 rpm for 10 minutes, and the thus collected cells were washed with 30 mM sodium chloride and a 30 mM Tris-HCl buffer (pH 7.5). The thus washed cells were suspended in 30 ml of the above-described buffer and disrupted ultrasonically at 0°C for 10 minutes using a sonicator (SINIFIER CELL DISRUPTOR
- 40 200, OUTPUT CONTROL 2, manufactured by BRANSON SONIC POWER COMPANY). The cell suspension after the ultrasonic disruption was centrifuged at 9,000 rpm for 30 minutes to obtain the cell residue.
- [0117] Thereafter, the hG-CSF derivative was extracted and purified from the cell residue and solubilized and regenerated, in accordance with the method of Marston *et al.* [F.A.O. Marston *et al.*, *BIO/TECHNOLOGY*, 2:800 (1984)].

45 Reference Example 2

Production of N-hydroxysuccinimide ester of 2,4-bis(o-methoxypolyethylene glycol)-6-(3-carboxypropylamino)-s-triazine:

- 50 [0118] 412 mg (4.0 mmol) of γ -aminobutyric acid was dissolved in 300 ml of a 0.1 M borate buffer (pH 10), and the resulting solution which was cooled in an ice bath was mixed with 20 g (2 mmol) of 2,4-bis(o-methoxypolyethylene glycol)-6-chloro-s-triazine (manufactured by Seikagaku Kogyo) and the mixture was allowed to react at 4°C for a whole day and night and then at room temperature for 6 hours.
- [0119] The reaction solution was adjusted to pH 1 by adding 1 N hydrochloric acid, and then 2,4-bis(o-methoxypolyethylene glycol)-6-(3-carboxypropylamino)-s-triazine was extracted with chloroform. The chloroform phase was dried
- 55 over anhydrous sodium sulfate, the insoluble matters were removed by filtration, and then the solvent was evaporated under reduced pressure to obtain the carboxylic acid derivative.
- [0120] The carboxylic acid derivative was recrystallized from dry acetone to obtain 15.8 g (1.6 mmol) of crystals of

the carboxylic acid derivative.

[0121] A 10 g (1.0 mmol) portion of the crystals and 230 mg of N-hydroxysuccinimide were dissolved in 100 ml of anhydrous methylene chloride and, while cooling in an ice bath and in a stream of argon, the resulting solution was mixed with 413 mg of N,N'-dicyclohexylcarbodiimide (DCC) and the mixture was stirred for 30 minutes. After completion of the stirring, the temperature of the reaction mixture was returned to room temperature, and stirred for additional 1.5 hours, the insoluble matters (N,N'-dicyclohexylurea (DCU)) were removed by filtration and then the resulting filtrate was concentrated to 40 ml under reduced pressure.

[0122] The concentrate was added dropwise to 600 ml of anhydrous diethyl ether to form a precipitate. The precipitate was washed with anhydrous diethyl ether and then the solvent was removed under reduced pressure to obtain 7.7 g (0.77 mmol) of N-hydroxysuccinimide ester of 2,4-bis(o-methoxypolyethylene glycol)-6-(3-carboxypropylamino)-s-triazine (yield, 77%).

Reference Example 3

Production of 2,4-bis(o-methoxypolyethylene glycol)-6-(3-carboxypropylamino)-s-triazine:

[0123] 100 g (8.33 mmol) of thoroughly dried monomethoxypolyethylene glycol having an average molecular weight of 12,000 (manufactured by Nippon Oil & Fats), 9.3 g of zinc oxide (Wako Pure Chemical Industries) and 83.5 g of molecular sieves (Type 4A) (Wako Pure Chemical Industries) were dissolved in dry benzene, and the solution was allowed to stand at room temperature for a whole day and night in a stream of argon.

[0124] After removing the molecular sieves, the reaction solution was distilled at 80°C in a stream of argon using a distillation apparatus and then azeotropically distilled for a whole day and night at 80°C in a stream of argon using a Soxhlet extractor (for solid phase use) which had been packed with about 100 g of molecular sieves (Type 4A).

[0125] The reaction solution obtained by the dehydration reflux was cooled, mixed with 736 mg (4.0 mmol) of cyanuric chloride and then azeotropically distilled for 5 days in a manner similar to that described above. Thereafter, this solution was cooled to room temperature, mixed with 300 ml of dry benzene and then the mixture was centrifuged at 3,600 rpm for 10 minutes to remove insoluble matter.

[0126] The thus obtained supernatant was concentrated to 300 ml under reduced pressure and added dropwise to 3,000 ml of dry diethyl ether to form a precipitate. The precipitate was recovered and washed with dry diethyl ether, and then the solvent was removed under reduced pressure to obtain a dry precipitate.

[0127] While cooling in an ice bath, 100 g of the dry precipitate was added to a solution prepared by dissolving 1.24 g (12.0 mmol) of γ -aminobutyric acid in 1,000 ml of a 0.1 M borate buffer (pH 10), and the mixture was stirred at 4°C for a whole day and night. After additional 6 hours of stirring at room temperature, this solution was adjusted to pH 1.0 with 1 N hydrochloric acid and then 2,4-bis(o-methoxypolyethylene glycol)-6-(3-carboxypropylamino)-s-triazine was extracted with chloroform.

[0128] The chloroform phase containing the compound was dried over anhydrous sodium sulfate and filtered, and then the solvent was removed from the resulting filtrate under reduced pressure. The thus formed white solid was recrystallized from dry acetone to obtain about 90 g of 2,4-bis(o-methoxypolyethylene glycol)-6-(3-carboxypropylamino)-s-triazine (yield, 90%).

Reference Example 4

Production of N-hydroxysuccinimide ester of 2,4-bis(o-methoxypolyethylene glycol)-6-(3-carboxypropylamino)-s-triazine:

[0129] 25 g (1.0 mmol) of 2,4-bis(o-methoxypolyethylene glycol)-6-(3-carboxypropylamino)-s-triazine which had been synthesized in a manner similar to that in Reference Example 3 and thoroughly dried and 240 mg of N-hydroxysuccinimide were dissolved in 400 ml of anhydrous methylene chloride and, while cooling in an ice bath and in a stream of argon, the resulting solution was mixed with 431 mg of N,N'-dicyclohexylcarbodiimide (DCC) and the mixture was stirred for 30 minutes.

[0130] After completion of the stirring, the reaction mixture was returned to room temperature and stirred for additional 1.5 hours, the insoluble matters (N,N'-dicyclohexylurea (DCU)) were removed by filtration and then the resulting filtrate was concentrated to 160 ml under reduced pressure.

[0131] The concentrate was added dropwise to 2,400 ml of anhydrous diethyl ether to form a precipitate, the precipitate was washed with anhydrous diethyl ether and then the solvent was removed under reduced pressure to obtain 21.4 g (0.89 mmol) of N-hydroxysuccinimide ester of 2,4-bis(o-methoxypolyethylene glycol)-6-(3-carboxypropylamino)-s-triazine (yield, 89%).

INDUSTRIAL APPLICABILITY

[0132] The present invention provides a chemically modified polypeptide in which at least one of the hydroxyl groups in the polypeptide molecule is modified with a polyalkylene glycol derivative; a method for producing the modified polypeptide; a method for treatment using the modified polypeptide; use of the modified polypeptide; a pharmaceutical preparation comprising the modified polypeptide; and a composition for treatment comprising the modified polypeptide.

Claims

1. A chemically modified polypeptide in which at least one of the hydroxyl groups in the polypeptide molecule is modified with a polyalkylene glycol derivative.
2. The chemically modified polypeptide according to claim 1, wherein the hydroxyl group in the polypeptide molecule is a hydroxyl group derived from a serine residue.
3. The chemically modified polypeptide according to claim 1 or 2, wherein the polypeptide is a polypeptide having a granulocyte colony-stimulating factor activity.
4. The chemically modified polypeptide according to claim 3, wherein the polypeptide having a granulocyte colony-stimulating factor activity is a polypeptide comprising the amino acid sequence represented by SEQ ID NO:1, or a polypeptide comprising an amino acid sequence in which at least one amino acid is deleted, substituted or added in the amino acid sequence represented by SEQ ID NO:1, and having a granulocyte colony-stimulating factor activity.
5. The chemically modified polypeptide according to claim 1 or 2, wherein the polyalkylene glycol derivative has a molecular weight of 500 to 1,000,000.
6. The chemically modified polypeptide according to claim 1, which is modified with a chemical modifying agent comprising a polyalkylene glycol derivative.
7. The chemically modified polypeptide according to claim 6, wherein the chemical modifying agent is a polyalkylene glycol derivative represented by the following formula (I):

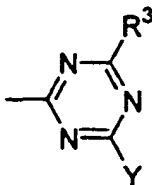


(wherein R^1 represents an alkyl group or an alkanoyl group; M represents

$-OCH_2CH_2-$, $-OCH_2CH_2CH_2-$ or

$-(OCH_2CH_2)_r-(OCH_2CH_2CH_2)_s-$

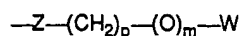
(wherein r and s are the same or different, and each represents an optionally changeable positive integer); n is an optionally changeable positive integer; X represents a bond, O, NH or S; and R^2 represents



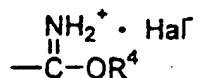
(wherein R^3 represents OH, halogen or



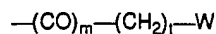
(wherein X^a , M^a , R^{1a} and n^a each has the same meanings as the above X, M, R^1 and n, respectively); and Y represents halogen or



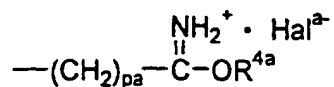
[wherein Z represents O, S or NH; and W represents a carboxyl group or a reactive derivative thereof, or



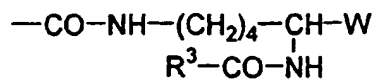
(wherein R^4 represents an alkyl group, and Hal represents halogen); p is an integer of 0 to 6; and m is 0 or 1],



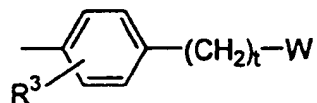
(wherein t is an integer of 0 to 6; and m and W have the same meanings as defined above),



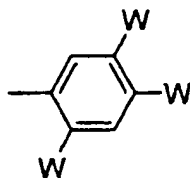
(wherein Hal^a , pa and R^{4a} each has the same meanings as the above Hal, p and R^4 , respectively),



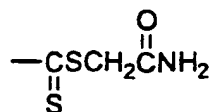
(wherein R^3 and W have the same meanings as defined above),



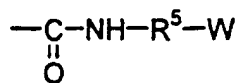
(wherein R^3 , t and W have the same meanings as defined above),



(wherein W has the same meaning as defined above),

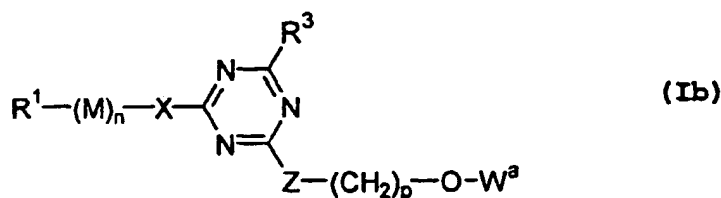


or



(wherein R^5 represents a residue in which an amino group and a carboxyl group are removed from an amino acid; and W has the same meaning as defined above)).

8. The chemically modified polypeptide according to claim 7, wherein the chemical modifying agent is a polyalkylene glycol derivative represented by the following formula (Ib):



(wherein R^1 , M, R^3 , Z, n and p have the same meanings as defined above; and W^a represents a carboxyl group or a reactive derivative thereof).

9. A method for producing a chemically modified polypeptide, which comprises reacting at least one of the hydroxyl groups in the polypeptide molecule with a chemical modifying agent comprising a polyalkylene glycol derivative.
10. A pharmaceutical preparation comprising any one of the chemically modified polypeptides according to claims 1 to 8.
11. A method for treating a patient having reduced granulocytes or thrombocytes, which comprises administering an effective amount of any one of the chemically modified polypeptides according to claims 1 to 8.
12. Use of any one of the chemically modified polypeptides according to claims 1 to 8 for the production of a pharmaceutical composition which is effective in treating a patient having reduced granulocytes or thrombocytes.
13. Use of any one of the chemically modified polypeptides according to claims 1 to 8 for the treatment of a patient having a reduced granulocytes or thrombocytes.
14. A composition for treatment of a patient having reduced granulocytes or thrombocytes, which comprises a pharmaceutically acceptable carrier and an effective amount of any one of the chemically modified polypeptides according to claims 1 to 8 in a pharmaceutically acceptable dosage form.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP98/02504

A. CLASSIFICATION OF SUBJECT MATTER Int.Cl ¹ C07K1/113, 14/535, A61K38/16, 38/19 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) Int.Cl ¹ C07K1/113, 14/00, A61K38/00 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CAS ONLINE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, 95/23165, A1 (Kyowa Hakko Kogyo Co., Ltd.), 31 August, 1995 (31. 08. 95), Pages 8 to 15 & EP, 744409, A1	1-14
Y	JP, 62-501449, A (University of New Mexico), 11 June, 1987 (11. 06. 87), Pages 2 to 4 & WO, 86/04145, A1 & US, 4732863, A	1, 2, 5-10
Y	JP, 3-95200, A (Sumitomo Pharmaceutical Co., Ltd.), 19 April, 1991 (19. 04. 91), Pages 4 to 6 & EP, 400472, A1 & US, 5342940, A	1, 2, 5-10
A	JP, 1-316400, A (Kyowa Hakko Kogyo Co., Ltd.), 21 December, 1989 (21. 12. 89), Pages 2 to 5 & EP, 335423, A1 & US, 5194592, A	1-14
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 26 August, 1998 (26. 08. 98)		Date of mailing of the international search report 8 September, 1998 (08. 09. 98)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.		Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

TD (Tina Darré)

From: mette@nettravel.dk
Sent: 26. marts 2001 13:14
To: td@maxygen.dk
Subject: Italien

Kære Tina

Jeg er nu ved at nå frem til et rigtig godt resultat vedr. din forespørgsel for gruppe rejse til Italien. Jeg satser nu på at sammensætte en pakke til Jer baseret på følgende :

Flyrejsen:
Jeg har fordelt Jer med 40 personer på SAS og 40 personer på Alitalia.

SAS til Bologna:			
SK1687	06SEP	København-Bologna	0855-1105
SK1688	10SEP	Bologna-København	1210-1415
Alitalia til Firenze via Milano:			
AZ129	06SEP	København-Milano	1155-1410
AZ1694	06SEP	Milano-Firenze	1530-1625
AZ1689	10SEP	Firenze-Milano	1155-1250
AZ128	10SEP	Milano-København	1350-1605

Men da jeg ikke selv mener at ankomst tiderne passer optimalt sammen har jeg også forespurgt Swiss Air / Sabena da de har en rigtig god forbindelse til Bologna. Jeg afventer deres svar senest i morgen da jeg har rykket kraftigt for svar i dag.

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SAS - per person: 2.350,- + lufthavnsskatter 245,- / total 2.595,-
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I mellemtiden ville jeg lige høre dig om alle 80 deltagere skal holde møde samtidig ? På forhånd tak.

Hvis du har nogle kommentarer eller spørgsmål til ovenstående er du meget velkommen til at kontakte mig.

Med venlig hilsen / With best regards

Mette Leonhardt Gartmann
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